ACID TOLERANCE AND ORGANIC ACID SUSCEPTIBILITY OF SELECTED FOOD-BORNE PATHOGENS

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ABSTRACT

The development of tolerance to low pH levels and the existence of cross-resistance may promote survival of bacteria in acidic foodstuff and in acidic environments such as the human stomach, in so doing escalating the probability of food poisoning. Similar to antimicrobial resistance developing, there is growing concern that effectiveness of organic acids may decrease as a result of the emergence of acid-tolerant food-borne pathogens. The objectives of this study were to determine the development of acid tolerance in selected food-borne pathogenic bacteria and to explore the activity of organic acids against acid tolerant pathogens. Bacterial strains were screened for acid-tolerance and susceptible strains were induced through exposure to increasing concentrations of an inorganic acid, as well as acidic foodstuffs. Susceptibility to six organic acids at various pH levels was assessed in order to evaluate the possible relationship between altered antimicrobial activity and acid tolerance. Salmonella enterica sv. Enteritidis ATCC 13076 and Escherichia coli ATCC 25922 were found to rapidly develop acid tolerance, while intrinsic acid tolerance was noted in Salmonella enterica sv. Typhimurium ATCC 14028. Pseudomonas aeruginosa ATCC 27853 demonstrated intermediate intrinsic acid tolerance. As expected, pH played a significant role in inhibitory activity of the organic acids as these compounds exhibit optimum antimicrobial activity at a lower pH (pH ≤5). It is, however, necessary to further elucidate the two-way role of pH in foodstuff concomitant to the addition of an organic acid.

Keywords: Organic acids, food borne pathogens, HCl, MIC

1. INTRODUCTION

The human gastric fluid plays an important role in first-line defense against enteric pathogens present in food by killing or inactivating these organisms before they enter the intestinal tract (Clark, 1999). However, when gastroenteritis does occur, it is necessary to determine whether pathogens ingested are acid-tolerant as well as the mechanism of such tolerance. Various foodstuffs, especially processed food, sauces and juices have a low pH, while bacteria have been reported to survive in such products. In cases where organic acids have been used as food preservatives, concern has been expressed that repetitive and prolonged exposure may result in the emergence of acid tolerant food-borne pathogens with the ability to overcome the protective barrier of the gastric environment.

Food-borne pathogenic bacteria exhibit various stress responses, which improve their continued survival in undesirable environmental conditions (Dilworth and Glenn, 1999). A universally encountered stress in foods is acidity, where survival may lead to the induction of an acid tolerance response (ATR) (Dilworth and Glenn, 1999). The ATR is defined as the resistance of organisms to reduced optimum pH levels when they have either been grown at moderately low pH or previously been exposed to sub-optimal pH levels (Dilworth and Glenn, 1999).

After acid adaptation, increased resistance to the inactivation of cells at lower pH has been reported in some studies of S. enterica serovar Typhimurium (Leyer and Johnson, 1993). The majority of studies concerning the development and evaluation of the ATR have been conducted at temperatures of 30 or 37˚C and made use of acidulates such as hydrochloric acid (HCl) in attempting to express the ATR response to conditions that are most relevant to food (Foster, 1991; Kroll and Patchett, 1992; O'Driscoll et al., 1996). Extensive studies on S. enterica serovar Typhimurium demonstrated that acid shock results in cross-protection against various environmental stresses (Foster and Hall, 1990; Foster and Hall, 1991; Foster, 1995), including environments related to fermented foods such as cheese (Leyer and Johnson, 1992). However, these studies utilised hydrochloric acid to lower pH levels abruptly, whereas lowering the pH in foodstuffs like yogurt and fermented meats occurs gradually as fermentation proceeds. To fully grasp the effect of acidic environments on acid tolerance of bacteria and their ability to survive in fermented and acidic foods, cells should be exposed to acidic environments by making use of methods that accurately simulate authentic food systems (Deng et al., 1999).

In food systems food-borne bacteria are generally in contact with weak organic acids. These acids may be intrinsically produced as a result of fermentation or added as food preservatives or sensory enhancements. Because organic acids are widely used in these processes, they are also appropriately suited to reduce pH in media (Deng et al., 1999).

Objectives of this study were to evaluate the development of acid tolerance in bacterial strains often associated with food-borne illnesses and to explore the possibility of repercussions in successful food preservation with organic acids. In designing the acid challenge methodology, both organic and inorganic acids were included in attempts to simulate acidic foodstuff as well as the human stomach environment.

2. MATERIALS AND METHODS

Bacterial isolates

Bacterial isolates consisted of species that have frequently been implicated in food-borne illnesses, resulting from the consumption of acidic foodstuffs (Alakomi et al., 2000).

These included standard bacterial strains E. coli ATCC 25922, P. aeruginosa ATCC 27853, S. enterica subsp. enterica sv. Typhimurium ATCC 14028 and S. enterica subsp. enterica sv. Enteritidis ATCC 13076.

Screening isolates for acid tolerance

The method described by Jordan et al. (1999) was adapted for the purpose of determining acid tolerance development. Isolates were cultivated in Mueller-Hinton (MH) broth (Biolab Diagnostics [Pty] Ltd., Auckland, NZ) (pH 7) for 48 h at 35˚C and consequently acid challenged through the reduction of the medium to pH 4.5 with HCl. Viable cell counts were determined prior to acid challenge and at consecutive intervals of 12, 24, 36 and 48 h after pH adjustment and expressed as CFU/ml. Serial dilutions were performed in 0.1% peptone, 10 µl aliquots spread-plated onto MH agar (Biolab Diagnostics [Pty] Ltd., Auckland, NZ) and incubated for 24 h at 35°C (Jordan et al., 1999).

Induction of acid tolerance

Bacterial strains were sub-cultured in Brain-Heart Infusion (BHI) broth containing increasing concentrations of hydrochloric acid as well as a variety of acidic foodstuffs (vinegar, mayonnaise, chopped gherkins and gherkin brine) and incubated at 30˚C for 24 h. Control broths were included for monitoring pH. Viable organisms at lowest pH levels for each induction medium (broth and foodstuffs) were inoculated onto BHI agar (pH 7 and pH 5) and incubated at 30˚C for 48 h. Acid-tolerant cells were harvested and stored at -80˚C.

Susceptibility testing

The minimum inhibitory concentrations (MICs) of the six organic acids for both the parent strains and induced strains were determined with an agar-dilution method, at various pH levels ranging from pH 5 to pH 8, as described by the Clinical and Laboratory Standards Institute (CLSI, 2006). Acetic acid, benzoic acid (sodium salt), lactic acid, malic acid, propionic acid, sorbic acid (potassium salt) were obtained from MP Biomedicals, Inc. (Solon, Ohio, USA). Cell suspensions were inoculated onto the surface of MH agar, containing doubling organic acid concentrations (ranging from 0.25-256 mM), using a multipoint inoculator (MultipointElite, Mast Laboratories, Merseyside, UK) to deliver 1 x 105 CFU per spot. After 24 h incubation at 35°C the MIC was recorded as the lowest concentration of organic acid where no growth was detected.

3. RESULTS AND DISCUSSION

The development of acid tolerance was noted in both S. enterica sv. Enteritidis and E. coli after 36 h of acid exposure. In S. enterica sv. Typhimurium intrinsic acid tolerance was evident, while P. aeruginosa did not show notable acquisition of acid resistance after exposure to pH 4.5. However, these organisms survived after 48 h, showing the same viable count as was recorded at 0 h (Figure 1).

Exposure to acidic foodstuff and also to HCl resulted in diverse susceptibility patterns to the organic acids (Table 1). Various changes in susceptibility in comparison to the unexposed strain were noted and these changes were not consistent among the bacterial strains. Of specific interest was P. aeruginosa, which displayed lower acid tolerance during the acid tolerance assay (Figure 1), but demonstrated decreased susceptibility to potassium sorbate, sodium benzoate, acetic acid and lactic acid after exposure to gherkin brine. P. aeruginosa actually revealed a wide variety of changes to organic acid susceptibility (Figure 2) and is the only strain that showed changes in MIC profiles at all pH values tested, although after exposure to different acidic foodstuffs. This trend was more visible after exposure to vinegar and gherkin brine.

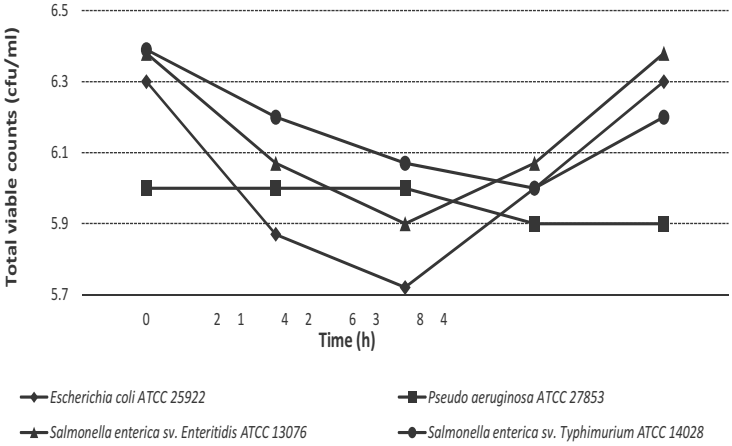

Figure 1: Total viable counts for Escherichia coli ATCC 25922, Salmonella enterica sv. Enteritidis ATCC 13076 and Salmonella enterica sv. Typhimurium ATCC 14028 and Pseudomonas aeruginosa ATCC 27853 after acid exposure

Contrary to these findings E. coli, S. enterica sv. Typhimurium and S. enterica sv. Enteritidis, strains which acquired acid tolerance more rapidly after acidic exposure, only showed significant changes in susceptibility to the organic acids at lower pH values (pH 5-6). E. coli showed differences only at pH 5 and 5.5 and more specifically to potassium sorbate (decreased susceptibility after exposure to all foodstuffs+HCl) and propionic acid (increased susceptibility after exposure to gherkin brine, mayonnaise and vinegar) at pH 5 and to malic acid (decreased susceptibility after exposure to gherkin brine, chopped gherkin and mayonnaise) at pH 5.5 (Table 1). At pH 5, S. enterica sv. Enteritidis showed increased susceptibility to propionic acid after exposure to gherkin brine and chopped gherkins, and decreased susceptibility to acetic acid after exposure to mayonnaise, vinegar and HCl.

S. enterica sv. Typhimurium showed changes in susceptibility (decrease) after exposure to all the foodstuffs and HCl only to acetic acid at pH 5 and to lactic acid at pH 6 and pH7 (Table 1). No changes in susceptibility were noted for S. enterica sv. Enteritidis, S. enterica sv. Typhimurium or E. coli (stressed or unstressed) when exposed to sodium benzoate (results not shown).

The susceptibility results demonstrated varying responses from the four different bacterial strains when exposed to low pH environments and acidic foodstuffs and no definite relationship was noted between the type of foodstuff and the effectiveness of a specific organic acid. It was however, evident that decreased susceptibility occurred in each organism to at least one organic acid tested and after exposure to at least one of the acidic foodstuffs. Greenacre et al. (2003) reported that by using either acetic or lactic acid for the exposure of S. enterica sv. Typhimurium to moderately pH levels at 20˚C, an ATR is notable where the adaptation time and pH represented the distinct conditions for ATR expression. The effect of a lowered internal pH (pHi) of bacterial cells led to an acid death, as proven by previous studies, but acid adapted cells were able to maintain their pHi more successfully, thus increasing their survival in an acidic environment (Foster and Hall, 1991; O'Driscoll et al., 1997).

The process of acid adaptation of microorganisms is complex and many physiological changes take place, including the expression of shielding stress proteins and even damage to cell membranes (Leyer and Johnson, 1993). The degree of acid tolerance is dependent on the nature of the physiological changes as well as the intensity of the subsequent stress factors. In some cases the effects of cellular damage might exceed the shielding effect of acid-shock proteins or other protective metabolic changes induced by low pH, and stressed cells could die if exposed to more harsh environments (Deng et al., 1999). It would be worth investigating possible alterations in the cell membrane proteins, specifically after acid induction.

Table 1: Susceptibility profiles of the various strains to the organic acids at various pH levels to acidic foodstuffs and HCl. Un = uninduced strain, GB = gherkin brine, CB = chopped gherkin, May = mayonnaise, Vin = vinegar, HCl = hydrochloric acid, SD = standard deviation, "-" = no change in MIC.

		Escherichia coli						*S. enterica* sv. Enteritidis						*S. enterica* sv. Typhi murium					
		pH						pH						pH					
		5.0	5.5	6.0	6.5	7.0	7.5	5.0	5.5	6.0	6.5	7.0	7.5	5.0	5.5	6.0	6.5	7.0	7.5
Potassium sorbate	**Un**	**8**	**32**	**64**	**128**	**256**	**256**	**8**	**16**	**64**	**128**	**>256**	**>256**	**16**	**64**	**64**	**128**	**>256**	**>256**
	GB	16	-	-	-	-	-	-	32	-	-	-	-	-	-	128	-	-	-
	CG	16	-	-	-	-	-	-	-	-	-	-	-	-	-	128	-	-	-
	May	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Vin	16	-	-	-	-	-	-	-	-	-	-	-	-	-	128	256	-	-
	HCl	16	-	-	-	-	-	-	-	-	64	-	-	-	-	128	-	-	-
Acetic acid	**Un**	**8**	**8**	**16**	**16**	**32**	**32**	**4**	**8**	**8**	**16**	**32**	**32**	**4**	**8**	**16**	**16**	**32**	**32**
	GB	-	-	-	-	16	-	-	-	16	-	-	-	8	-	-	-	-	-
	CG	4	-	-	-	16	-	-	-	16	-	16	-	8	-	-	-	-	-
	May	4	-	-	-	-	-	8	-	16	-	16	-	8	-	-	-	-	-
	Vin	4	-	-	-	-	-	8	-	16	-	-	-	8	-	-	-	16	-
	HCl	-	-	-	-	-	-	8	-	16	-	-	-	8	-	-	-	16	-
Lactic acid	**Un**	**64**	**64**	**64**	**128**	**256**	**>256**	**64**	**64**	**64**	**128**	**128**	**>256**	**64**	**64**	**64**	**128**	**256**	**>256**
	GB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	128	256	-	-
	CG	-	-	-	-	-	-	-	-	128	256	256	-	-	-	128	256	-	-
	May	-	-	-	-	-	-	-	-	128	256	256	-	-	-	128	256	-	-
	Vin	-	-	-	-	-	-	-	-	-	-	256	-	-	-	128	>256	-	-
	HCl	-	-	-	-	-	-	-	-	128	-	256	-	-	-	128	256	-	-
Malic acid	**Un**	**16**	**16**	**32**	**32**	**32**	**32**	**32**	**32**	**64**	**32**	**64**	**64**	**32**	**32**	**64**	**32**	**64**	**64**
	GB	-	32	-	-	-	-	-	-	-	64	-	-	-	-	-	64	-	-
	CG	-	32	-	-	-	-	-	-	-	64	-	-	-	-	-	-	-	-
	May	-	32	-	-	-	64	-	-	-	64	-	-	-	-	-	64	-	-
	Vin	-	-	-	-	-	-	-	-	-	64	-	-	-	-	-	64	-	-
	HCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	64	-	128
Propionic acid	**Un**	**8**	**8**	**16**	**16**	**32**	**32**	**8**	**8**	**8**	**16**	**32**	**16**	**2**	**4**	**8**	**16**	**16**	**16**
	GB	4	-	-	-	-	-	4	-	16	-	-	-	-	-	-	-	-	-
	CG	-	-	-	-	-	-	4	-	16	-	-	-	-	-	-	-	-	-
	May	4	-	-	-	-	-	-	-	16	-	-	-	4	-	-	-	-	32
	Vin	4	-	-	-	-	-	-	-	16	-	-	-	-	-	-	-	-	-
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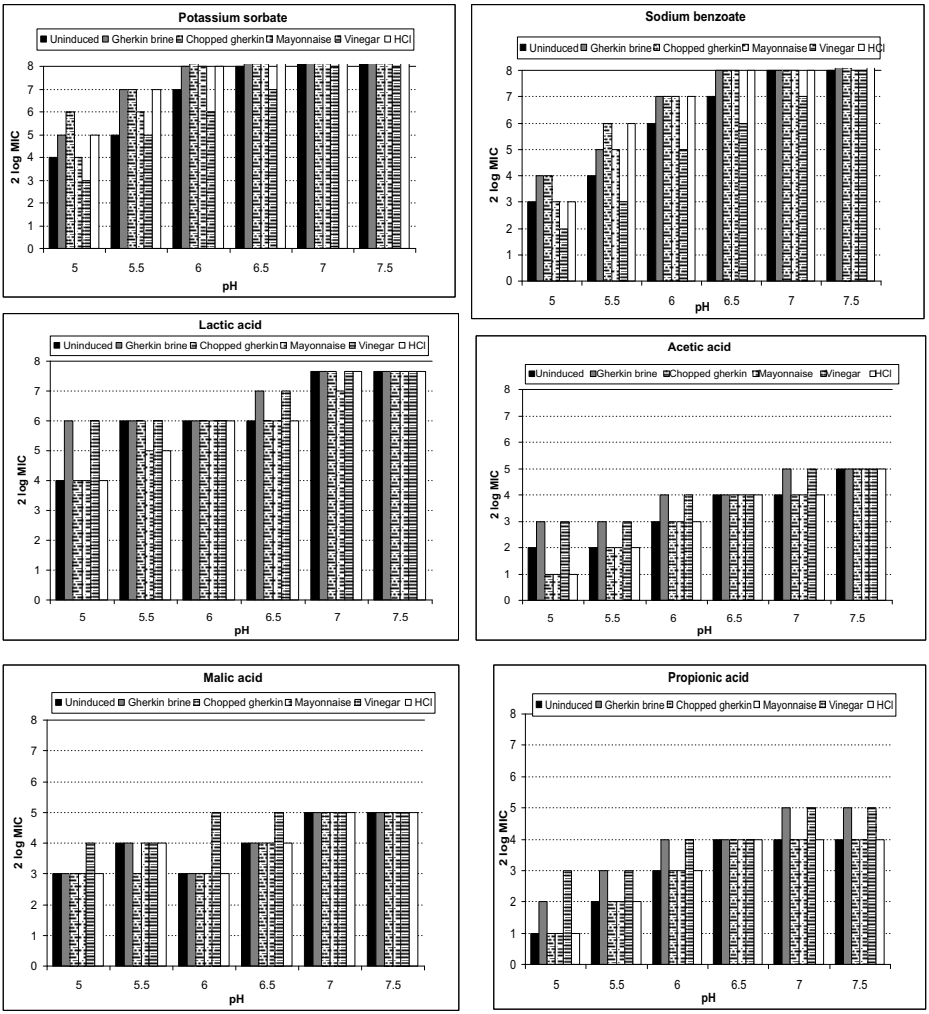

Figure 2: Susceptibility profiles of P. aeruginosa ATCC 27853 to organic acids at various pH profiles to acidic foodstuffs and HCl.

4. ACKNOWLEDGEMENTS

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